Perspective

Engineering Challenges of Protein Formulations

Theodore W. Randolph
Dept. of Chemical and Biological Engineering, University of Colorado, Boulder, CO 80309

John F. Carpenter
Dept. of Pharmaceutical Sciences, University of Colorado Health Sciences Center, Denver, CO 80262

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Introduction

Protein based pharmaceuticals are the fastest growing class of new drugs. They not only offer promise for treatments to address major health challenges, such as cancer, but also a wealth of new engineering problems to solve. Chemical engineers have long been proficient at pro ducing products that meet exacting specifications for chemical purity, but therapeutic proteins now bring additional chal lenges: these products must not only be highly chemically pure, but also *conformationally* pure, and must remain so dur ing manufacturing and through the drug's entire shelf life and delivery to patients.

Proteins degrade through a variety of mechanisms. These are usually classified as either physical or chemical degradation pathways.[1] Physical degradation pathways include unfolding, misfolding, and aggregation of the protein molecules. Chemical degradation pathways encompass a myriad of unwanted chemical reactions that proteins commonly undergo, such as oxidation, deamidation, racemization, hydrolysis, disulfide exchange, and carbamylation. Classification of degradation pathways as physical or chemical is somewhat artificial, because the two types of degradation often are closely linked. For example, we have shown that an oxidation process result ing in crosslinking of tyrosine residues in α synuclein (a protein that forms characteristic fibrils in Parkinson's disease), is a pre cursor to aggregation and fibrillogenesis.[2] Addition of radical scavenging molecules, such as methionine to α synuclein for mulations delays onset of *in vitro* fibril formation by reducing the rate of tyrosine oxidation formation. Conversely, oxidation of the serine protease subtilisin can be inhibited by adding for mulation excipients, such as sucrose that act to increase the thermodynamic conformational stability of the protein.[3]

Correspondence concerning this article should be addressed to T. W. Randolph at theodore.randolph@colorado.edu.

Formulation Challenge

To allow proteins to be used as therapeutic agents, proteins must be placed in a formulation that confers suitable stability against physical and chemical degradation. In addition to sta bilizing the pharmaceutically active protein ingredients, for mulation components, or excipients, also must be compatible with their intended use. For example, a formulation intended for parenteral use (e.g., subcutaneous injection) must be ster ile, nontoxic, and exhibit acceptable viscosity and tonicity. Although these requirements place limits on the types and concentrations of excipients that practically can be used, there are still far too many possible sets of formulations to allow a purely empirical screening approach to be successful.

For economic viability, therapeutic protein formulations typically require a shelf life of 18 24 months.[4] Over the course of this time, when stored as directed on the product label (usually refrigerated at 2 8 °C), the protein must retain adequate chemical and conformational purity. Meeting the stringent requirements for stability during shelf life is a daunt ing task. Most of the common chemical degradation products (especially hydrolysis and oxidation byproducts) are signifi cantly thermodynamically favored vs. the desired native state of the protein. Furthermore, the properly folded native state of most proteins is only marginally more stable (the free energy of unfolding ΔG_{umf}, is about 20 60 kJ/mol) than the folded state,[5] and appears to be *unstable* under most conditions with respect to aggregated forms of the protein.[6,7]

Required chemical and conformational purity levels are dic tated by the individual protein's safety and efficacy profile, but frequently levels of chemical impurities >5%, or confor mational impurities >1% at the end of the labeled shelf life might be considered unacceptable. Given that typical concen trations of protein in therapeutic formulations are near 10 micromolar, this suggests that levels of degradation prod ucts typically must be held below 100 nanomolar over the course of two year storage. Thus, an average rate of degrada

tion of 1 nanomolar/week may indicate an unacceptable level of product lability.

As part of the approval process for protein therapeutics, the US Food and Drug Administration requires that protein drug stability be demonstrated in real time, under conditions mimicking the proposed labeled storage conditions, i.e., in the proposed container/closure system, at the proposed protein concentration in a final formulation and under specified temperature conditions. This requires that enormous resources be dedicated years before a product can be sold, and represents a bottleneck for the entire therapeutic protein development process. For products with anticipated annual sales often of more than $500M, delays in development of suitably stable formulations may, thus, represent lost sales of $1M or more per day. Clearly, a goal is to make sure that the formulations that enter real time stability testing have a high probability of success.

For many kinds of protein degradation, acceptable levels of degradation products at the end of shelf life are very low. This creates a quandary. We wish to be reasonably sure at the onset of a real time stability study that at the study's completion 18 or 24 months later we will have acceptably low levels of degradation products. However, in part, because of analytical limitations, it is difficult or impossible to conduct a relatively short (e.g., one week) experiment under proposed storage conditions that can be extrapolated to an 18 or 24 month storage life. Thus, in order to predict which set of excipients are likely to provide an adequate shelf life, accelerated degradation experiments must be conducted. In these experiments, formulations are subjected to an additional "stress", such as elevated temperature, freeze thawing, the presence of air water interfaces or high or low ionic strengths, and the kinetics of protein degradation measured. Combinations of excipients that protect against degradation under "stressed" conditions are then assumed to be most likely to confer stability under more benign storage conditions. Usually, the result of such studies is a formulation that provides the greatest relative stability. However, there is no assurance that this level of stabilization will be sufficient for the shelf life.

By definition, accelerated degradation studies are conducted under conditions that are different from anticipated actual storage conditions. How predictive are these studies of protein behavior at actual storage conditions? fortunately, the answer (at least for relatively simple accelerated stability studies) is often "not very". Discrepancies between the predictions of simple accelerated studies and actual behavior might not be surprising, given the complicated structure of proteins, and the likely presence of multiple degradation pathways, but they serve to emphasize the need for better models and mechanistic understanding of protein degradation. Significant progress has been made in the "rational design" of protein formulations,[4,8], but there remain lacunae in the mechanistic understanding of the protein degradation pathways, and their responses to accelerated stability protocols. Some selected examples of challenges and progress in the protein formulation arena follow.

Thermally accelerated protein degradation

Elevated temperature is perhaps the most obvious "stress" condition that might be applied to accelerate protein degradation. Intuitively, one might expect that a simple Arrhenius analysis might allow data obtained on protein degradation at elevated temperatures (e.g., rate constants for protein aggregation measured at 50 80 °C) to be extrapolated to typical refrigerated storage conditions. However, often it is found empirically that predictions made using such an approach are poor. A reason for the observed discrepancy between predictions of degradation rates, based on simple Arrhenius analysis of thermally accelerated stability studies and actual behavior, lies in the coupling of thermodynamic equilibria between various protein conformations, each with a characteristic reactivity for a given degradation pathway and the kinetics of reactions on that pathway. Some protein degradation pathways, notably those leading to aggregation, occur through partially unfolded intermediates or through reactive subpopulations of the protein's native state ensemble. Because of the marginal conformational stability of proteins, relatively small changes in temperature can significantly perturb conformational stability, which in turn alters the population of aggregation prone protein molecules. For example, Roberts[9] has shown that the rate of aggregation of recombinant bovine granulocyte colony stimulating factor as a function of temperature, shows strikingly non Arrhenius behavior and a simple prediction of shelf life based on a simple Arrhenius extrapolation of data taken above room temperature to 5 °C storage conditions, would lead to an overestimation of shelf life by orders of magnitude. However, when the temperature dependency of recombinant bovine granulocyte colony stimulating factor conformational stability, and its effect on the observed rate constants were taken into account, the underlying rate constants for aggregation showed Arrhenius behavior.[9]

A commonly used approach to screen excipients for protein formulations is to use differential scanning calorimetry to measure the apparent "melting temperature" T_m, or temperature at which the protein olds in a given formulation. Formulations that yield elevated values are those in which the protein is presumed to be the most stable under real time storage conditions.[10] However, there are some excipients (notably nonionic surfactants and some preservatives) that lower values, but have little detrimental effect or act to increase stability at lower temperature storage conditions. For example, in the presence of the preservative benzyl alcohol, the apparent of recombinant human interleukin 1 receptor antagonist is depressed by about 8 °C, and the protein aggregates rapidly at 37 °C. However, little effect of benzyl alcohol is seen on the aggregation rate 25 °C.[11] In part, the contradiction can be explained on the basis of the temperature dependency of hydrophobic interactions, which are strengthened at higher temperatures. At higher temperatures, increased hydrophobic interactions favor binding of preservative to the exposed hydrophobic regions of olded protein molecules, which, according to the Wyman linkage function,[12] should result in a greater population of olded species, and, hence, a lower T_m than in the absence of preservative.[11]

Protein aggregation frequently appears to result from multistep and/or multipathway reactions.[13–15] Because the activation energies for each step in the reaction pathway may be different, the rate limiting step for the degradation process may change with temperature. In the case of proteins that exhibit multiple degradation pathways, the dominant degradation product that is formed during storage at refrigerated conditions may be different than that formed at temperatures used for accelerated stability studies. For example, when stored at

temperatures near room temperature, interleukin 1 receptor antagonist forms irreversible, soluble aggregates with nearly native secondary structure that are crosslinked through disul fide bonds.[16] In contrast, under accelerated degradation condi tions at 55 °C, the protein forms aggregates that are not cross linked through disulfides, but that contain significant non native β sheet structures.[17] Clearly, in this case extrapolations of protein aggregation kinetics based on high temperature studies would not be expected to be predictive of low temper ature storage behavior.

Pressure is a variable that may provide a useful alternative to temperature for accelerated stability studies. Analogous to using Arrhenius plots to determine activation energies, semilo garithmic plots of reaction rate constants vs. pressure may be used to determine the activation volume for a reaction. Acti vation volumes may then be used to predict rate constants at other pressures of interest. Webb et al.[18] measured activation volumes for aggregation of interferon γ, and found that the volume change required for aggregation −41 mL/mol, was only about 20% of the volume change required for complete unfolding of interferon γ (−209 mL/mol), suggesting that the reactive species involved in the aggregation of interferon γ is a partially unfolded,[19] rather than a completely olded spe cies.[18] Additional measurements of folding equilibria and aggregation kinetics made as a function of temperature and surface tension also show that the transition state for aggrega tion of interferon γ is partially, rather than fully unfolded (Figure 1). In the case of interleukin 1 receptor antagonist, activation volumes for aggregation were nearly identical to those required to old the protein, suggesting that a nearly com pletely olded species was required for aggregation. Interest ingly, the degradation products were disulfide bonded dimers similar to those seen after long term storage at atmospheric pressure and temperatures near room temperature.[17]

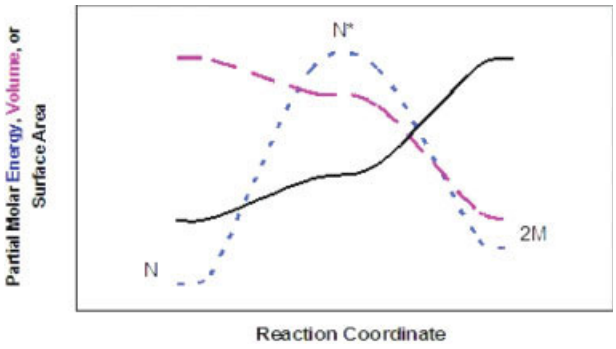

Figure 1. Reaction coordinate for aggregation of interferon- at 32 °C.

Interferon γ, a protein whose native state is a homo dimer, unfolds and aggregates rapidly upon dissocia tion into monomers. When the transition state is formed from the native state, the protein's partial molar volume (magenta) decreases by 41 mL/mol, the partial molar solvent exposed surface area increases (black) by 3.5 nm^2/molecule, and the associated activa tion energy E_a (blue) is 130 kJ/mol. In contrast, com plete dissociation and unfolding of the native state dimer is associated with a partial molar volume decrease of 209 mL/mol, a partial molar solvent exposed surface area increase of 12.7 nm^2/molecule, a free energy change of 27.2 kJ/mol, and an enthalpy change ΔH of 460 kJ/mol.[1]

Mechanisms of Protein Aggregation

Proteins are highly susceptible to the formation of non native aggregates and precipitates.[20,21] Irreversible, non native protein aggregation is a ubiquitous concern for biopharma ceuticals and biotechnology products,[22] because the biological activity of a protein in an aggregate is usually greatly reduced. More importantly, non native protein aggregates can cause adverse reactions in patients, ranging from immune response to anaphylactic shock and even death.[23–25] Adverse responses to aggregates of a given protein cannot be predicted, nor can the maximal level of aggregates that can be safely tolerated be determined without costly and time consuming clinical tri als.[4] Unfortunately, the link between immunogenicity and pro tein aggregates is often not discovered until side effects are noted, following either long term administration or increases in the patient population size after the drug has been approved. Thus, it is essential during product development to design proteins and protein formulations that minimize protein aggregation.

Protein aggregates generally exhibit secondary structures that are rich in β sheet structures, and that are dramatically perturbed from the protein's native secondary structure.[26] Pro tein aggregation rates depend strongly on protein conforma tion,[27] and even relatively small perturbations in protein struc ture can be sufficient to form transition state species on the aggregation pathway.[18,19] The kinetics of protein aggregation are controlled by both the concentration and the reactivity of these partially unfolded, transient intermediate species. If we assume that the free energy change associated with partial unfolding to form an aggregation competent transition state (ΔG^*) is of the same order of magnitude as that for complete unfolding (20 60 kJ/mol), on average fewer than 1/10,000 of the protein molecules exist in the transition state, or about 3 nM at typical formulation conditions. This creates an experi mental challenge, because the concentrations of these transient species are too low for direct measurement.

Although the properties of proteins in the aggregation competent transition state cannot be accessed spectroscopi cally, some insight into how formulation conditions affect aggregation rates can be gained by making two assumptions. The first assumption is that ΔG^* and ΔG_{umf}, are positively correlated. Thus, measurements of excipient effects on ΔG_{umf}, which can be made using various calorimetric and spectro scopic techniques.[28] should allow at least a qualitative predic tion of excipient effects on ΔG^*, and excipients that stabilize the native state against unfolding and increase $\Delta G_{umf,}$ should also reduce the equilibrium concentrations of partially unfolded aggregation competent species. A second assump tion is that the dominant protein protein interactions between native proteins are similar to those between protein molecules in the transition state. Protein protein interactions can be quantified by measurement of the osmotic second virial coeffi cient B_{22}.[14] Large, positive B_{22} values reflect net repulsive interactions between protein molecules. Formulation strat egies may, thus, be designed so as to reduce protein aggrega tion by adding using solution conditions (e.g., pH), and exci pients that increase ΔG_{umf} and/or B_{22} values.[14] Examples of protein formulations that have been stabilized by addition of agents that increased ΔG_{umf} include stabilization of acidic fibroblast growth factor,[29] and recombinant keratinocyte

growth factor[30] by polyanionic excipients, stabilization of recombinant human growth hormone and recombinant human nerve growth factor by addition of zinc,[31] and stabilization of recombinant human interferon γ by addition of sucrose.[27] Commercial formulations of recombinant human granulocyte colony stimulating factor, in contrast, minimize aggregation by adopting the strategy of maximizing protein protein repul sive interactions by formulating at low pH, where B_{22} values are large and positive.[14]

Formulations at High-Protein Concentrations

Many of the first generation of recombinant protein ther apeutics, such as erythropoietin and interferon γ, are extremely potent molecules that required only minute amounts of protein per dose. For example, erythropoietin is administered in a dosage form containing about 60 μg/mL erythropoietin. In contrast, newer, antibody based products are less potent, and, hence, require much larger doses. For example, the monoclonal antibody Herceptin® is sold in a vial containing 440 mg protein. The requirement for nearly 10,000 fold increases in protein dosages, combined with practical limitations on the volume (<1.5 mL) that can be delivered in a single subcutaneous dose has led to the need to develop formulations that are highly concentrated in protein.

Development of these formulations poses a number of seri ous obstacles to commercialization.[32,33] Although protein con centrations rarely exceed 10 mM, even in highly concentrated formulations, due to the relatively large molecular weight of proteins, this may represent a substantial volume fraction (10 15%) of the formulation. Solution nonidealities caused by protein protein interactions in these solutions may result in undesirably high solution viscosities,[32] opalescence,[34] and increased rates of aggregation.[35] High viscosities are problem atic because they can make manufacturing operations, such as filtration impractical, or limit the ability to deliver doses via narrow bore syringe needles. Opalescence, although not nec essarily harmful in itself, compromises the ability to detect product aggregation or particulate contamination within a vial, and creates difficulties during clinical trials, because of the lack of availability of opalescent placebo solutions. Most com mon analytical techniques used to examine protein protein interactions have been developed for use with much lower protein concentrations, and so current understanding of the interactions that cause high viscosities or opalescence concen tration is limited in part by the lack of appropriate analytical technologies.

In recent unpublished studies, we have shown that sim ple Carnahan Starling hard sphere models[36] of protein ac tivity coefficients accurately predict the protein concentra tion dependence of apparent rate constants for dimerization of recombinant human interleukin 1 receptor antagonist. In contrast, the effect of protein concentration on solution vis cosities for the same protein are poorly predicted from hard sphere models. A detailed understanding of the pro tein protein interactions that cause viscosity to vary from those predicted form hard sphere models is not available at this time.

Heterogeneous Nucleation during Processing and in Final Product Containers

Even under solution conditions where protein physical sta bility appears to be optimized so as to minimize protein aggregation in the bulk solution there can be formation of visible and subvisible protein particles that may constitute only a minute fraction of the total protein population. The presence of even a small number of protein particles can render a product clinically unacceptable. Particle formation can occur routinely during processing steps, such as pumping of protein solution during vial/syringe filling. In other cases, particle formation may appear to be random. For example, particles will be seen in a small fraction of vials or prefilled syringes in a given product lot. Unfortunately, these particles formed during vial filling operations appear downstream of sterile filtration steps and practically cannot be removed by fil tration.

We hypothesize that protein particle formation can arise from heterogeneous nucleation of protein aggregation on the surfaces of microparticles of foreign materials. These particulate contaminants can include metals shed from vial filling pumps, tungsten microparticles produced during man ufacture of glass syringes, and glass microparticles shed from vials as a result of high temperature depyrogenation procedures. Although such particles and the protein aggre gates that we hypothesize result from them are ubiquitous, virtually no systematic characterization of the problem, and the mechanisms governing it have been addressed in the literature. Aggregation at microparticle surfaces has been studied only for a limited number of surfaces and proteins, and under an even more limited range of solution condi tions, with few of the tested conditions being relevant for parenteral formulations of therapeutics. Furthermore, only two published studies have focused on therapeutic pro teins,[37,38] and none have focused on monoclonal antibodies, which are the largest class of therapeutic products currently being tested clinically.

It should be noted that for a commercial pharmaceutical product, usually it is not economically practical to eliminate the risk of heterogeneous nucleation by re engineering the sur face properties of containers, pumps or tubing to completely eliminate shedding of particles, or by re engineering the pro tein to reduce interactions with a surface. In fact in a recent review chapter Akers and Nail state, “Regardless of the qual ity of glass, the reputation of the manufacturer, the method of manufacture, or the method of cleaning, glass particulates are unavoidable.”[39] Thus, development of a safe, effective formu lation of a protein therapeutic depends on determining solu tion conditions that prevent the interactions of proteins with microparticulate contaminants that nucleate formation of pro tein particles, while still maintaining protein stability in bulk solution. However, current commercial formulation develop ment[8] does not include testing for heterogeneous nucleation during processes, such as vial filling. This problem is not triv ial. For example, relatively high concentrations of nonionic surfactant may reduce protein binding to contaminants, but could also foster unacceptably rapid aggregation of the protein in bulk solution.

Numerous studies have investigated the effects of surface chemistry on protein adsorption (see, for example[40], and refer ences therein). A major motivation of these earlier studies has been trying to understand the roles of protein interactions with surfaces associated with implantable medical devices. Conse quently, the solution conditions for these investigations have generally been chosen to mimic physiological conditions, e.g., phosphate buffered saline, and the surfaces tested have often been those characteristic of implantable devices or natural sur faces found *in vivo*, such as bone. In contrast, for formulations of therapeutic proteins, solution conditions are typically cho sen that are not physiological, but rather optimized to provide long term storage stability to the protein. For example, Amgen's recombinant human granulocyte colony stimulating factor product is formulated in HCl, pH 4.0, a solution condi tion that provides two year shelf life for the protein (in con trast, the protein forms aggregates within a week if stored in phosphate buffered saline at pH 7). Thus, the effects of solu tion conditions (especially, the role of pharmaceutical exci pients required for control of tonicity, antimicrobial activity, or protein stability in bulk solutions) on protein interactions with foreign microparticles have received limited attention in the literature.

In previous studies of protein aggregation in solution, we found that the rate of aggregation can be manipulated by alter ing solution conditions to modify protein conformational and colloidal stability.[14] However, for cases where heterogeneous nucleation is operative, it is unclear whether control of these factors is sufficient to prevent protein aggregation. For example, in our studies of protein recombinant human platelet activating factor acetylhydrolyase, we observed significant protein particle formation, even in formulations that conferred both conforma tional and colloidal stabilities.[37] We traced the cause of particle formation to the presence of small numbers of glass micropar ticles that were present in the drug product vials, presumably created during commercial depyrogenation procedures.

Conclusions

The remarkable advances in proteomics, development of fully humanized monoclonal antibodies and rapid drug candi date screening have led to a large number of proteins that are under development as possible therapeutics. Development of stable, pharmaceutically acceptable formulations now poses a bottleneck that must be addressed if we are to take full advantage of these remarkable new drug candidates. The cou pling of conformational equilibria with reaction kinetics, under solution conditions that tax existing analytical techni ques will provide chemical engineers and pharmaceutical sci entists with challenges for some time to come.

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